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Adherence of *Pseudomonas aeruginosa* to Tracheal Cells Injured by Influenza Infection or by Endotracheal Intubation

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Adherence of *Pseudomonas aeruginosa* to normal, injured, and regenerating tracheal mucosa was examined by scanning electron microscopy. Uninfected and influenza-infected murine tracheas were exposed to six strains of *P. aeruginosa* isolated from human sources and one strain of plant origin. All of the strains tested adhered to desquamating cells of the infected tracheas, but not to normal mucosa, the basal cell layer, or the regenerating epithelium. Adherence increased when the incubation time of the bacteria with the trachea was prolonged. Strains isolated from human tracheas appeared to adhere better than strains derived from the urinary tract. After endotracheal intubation of ferrets, *P. aeruginosa* adhered only to the injured cells and to areas of exposed basement membrane. We call this phenomenon "opportunistic adherence" and propose that alteration of the cell surfaces or cell injury facilitates the adherence of this bacterium and that adherence to injured cells may be a key to the pathogenesis of opportunistic *Pseudomonas* infections.

Bacterial adherence to host tissues is a primary step in the colonization and infection of mucosal surfaces (6). This may be especially true of mucosal surfaces where fluid flow and mechanical factors facilitate the removal of microorganisms. The adherence of pathogenic bacteria to the gastrointestinal (5, 10) and the genitourinary tracts (13, 14) has been reported, but there are few examples of this phenomenon occurring in the respiratory tract. *Mycoplasma pneumoniae* (12) and *Bordetella pertussis* (3) have been observed to adhere to tracheal cells, but this phenomenon has not been reported with the more common bacterial pathogens of the respiratory tree such as the *Pneumococcus, Haemophilus influenzae, Staphylococcus aureus* and the aerobic gram-negative rods.

The normal respiratory tract efficiently removes bacteria that have been aspirated into the airways. In fact, it is extremely difficult to produce respiratory disease experimentally in animals by the aspiration of microorganisms, and often some form of injury to the respiratory tract is required (1). Similarly, human bacterial respiratory disease often requires a predisposing insult to the tracheobronchial tree. Viral infections (4, 11) endotracheal intubation (8), and chemical injury (2) are some of the recognized antecedents of bacterial respiratory infections. In light of the association between these various insults and respiratory disease, we have studied the adherence of bacteria to the normal and injured tracheobronchial tree. In this report, we demonstrate that *Pseudomonas aeruginosa* adheres to injured tracheal cells after influenza virus infection and endotracheal intubation.

MATERIALS AND METHODS

Animals. Male mice of the AJ strain were obtained from Jackson Laboratories, Bar Harbor, Maine, at 5 to 6 weeks of age and used in the viral infection experiments at 8 weeks of age or older. Adult ferrets were obtained from Marshall Research Animals, Inc., North Rose, N.Y. for use in the intubation experiments.

Virus. A strain of A/Port Chalmers/1/73 influenza A virus, passaged in eggs since isolation and passed in AJ Swiss white mice twice for adaptation to the tracheobronchial tree, was used as the infecting viral agent. The virus preparation was titrated by egg infectivity, i.e., 50% egg infectious doses. An inoculum of $10^{4.5}$ 50% egg infectious doses was administered to mice intranasally after light ether anesthesia.

Bacterial strains. Six strains of *P. aeruginosa* were obtained from the Shands Teaching Hospital Clinical Microbiology Laboratories, three isolated from tracheal secretions of three different patients, and three from the urinary tracts of different patients. All strains were collected at different times and were transferred from the original plates onto Trypticase agar slants. One strain, isolated from a plant, was obtained from the Plant Pathology Department of the University of Florida.

Procedure. The strains of *P. aeruginosa* were cultivated overnight in Tryptic soy broth (Difco Laboratories, Detroit, Mich.) without agitation in a 37°C incubator. Organisms were centrifuged for 15 min at 12,500 × *g* and then suspended in phosphate-buffered saline (pH 7.4). The suspension was standardized by

optical density to give approximately 10^9 organisms per ml. Mice which were infected 2, 3, or 6 days previously with influenza virus and uninfected mice were anesthesized with sodium pentobarbital (0.06 mg/g intraperitoneally, Nembutal, Abbot Laboratories) and exsanguinated, and the tracheas were exposed in situ. A volume of 0.03 ml of the bacterial suspension was injected into the tracheal lumen after insertion of a 27-gauge needle through the upper end of the larynx. Animals were placed in a 37°C incubator for 15, 45, or 120 min. After removal from the incubator, one lung was excised to provide drainage and 0.3 ml of phosphate-buffered saline was slowly injected through the larynx to wash out nonadherent bacteria. The tracheas were then dissected from the thoracic cavities and placed in a 2.5% glutaraldehyde-cacodylate buffer solution (pH 7.4) for fixation. After fixation for 24 h or more, the tracheas were bisected longitudinally. One half of each trachea was dehydrated in ascending concentrations of acetone (70 to 100%), and critical-point dried in a Bomar SPC/900Ex critical-point drying machine (Bomar Corp., Tacoma, Wash.), with liquid carbon dioxide as the transitional fluid. After being mounted on aluminum studs, the specimens were sputter coated with gold-palladium in a Hummer II sputter coating machine (Technics, Alexandria, Va.) and examined with a Novascan 30 electron microscope (Semco, Ottawa, Ontario). Samples were examined to determine (i) which cells had adherent bacteria and (ii) how many bacteria were attached to a cell. After it was observed that bacteria adhered only to desquamating cells, 100 such cells were examined by traversing the mucosal surface in a preplanned manner, using the coordinates on the scanning electron microscope stage. It was often necessary to examine two or three tracheas to find 100 desquamating cells.

Ferrets were anesthesized by the intramuscular administration of ketamine hydrochloride (60 mg/kg; Ketaset, Bristol Laboratories, Syracuse, N.Y.) and a size 12 French-cuffed endotracheal tube (American Hospital Supply, Evanston, Ill.) was inserted into the trachea by using a laryngoscope. The endotracheal tube was inflated to a pressure of 25 cm of water and left in place for 2 h, and the animal was sacrificed. The thoracic cavity of the animal was opened. The area of tube placement was demarcated with a Sharpie marker (Sanford Corp., Bellwood, Ill.), and the trachea was then removed. This area and another area below the site of tube placement (control) were used in adherence experiments. Sections of trachea (ca. 0.5 by 0.5 cm) were excised from these areas, and bacterial suspensions were placed on the mucosal surfaces. These sections were then placed in small covered Petri dishes in a 37°C incubator for 45 min. After incubation they were washed by dipping them into three changes of phosphate-buffered saline, fixed and prepared for scanning electron microscope examination as described above.

RESULTS

Mouse trachea. Influenza infection produced desquamation of the tracheal surface. The uninfected trachea was not desquamated.

(i) Uninfected mouse trachea. Seven strains of *P. aeruginosa* were tested for adherence to normal mouse trachea. Each strain was tested in at least three mouse tracheas with incubation periods of 15, 45, and 120 min in vivo. Of the seven strains, none adhered in large numbers to the normal mucosa, and when bacteria were found they were single (Fig. 1A). Less than 10 organisms were found after examining about 3 mm^2 of each tracheal surface.

(ii) Influenza-infected mouse trachea. In contrast to the uninfected mouse trachea, the infected trachea proved to be a good nidus for adherence (Fig. 1B and C). *P. aeruginosa* was found adhering to desquamating or desquamated mucosal cells which were lying on the tracheal surface, although not all of these cells had adherent bacteria. The numbers of bacteria per cell (of those cells which had bacteria attached) varied from one to innumerable (Fig. 1B and C). Bacteria adhered to both ciliated and nonciliated cells, but only on days 2 and 3 after influenza virus infection. At these times, desquamating cells were usually still present on the tracheal surface, although fewer cells were present on day 3 than on day 2. Contrary to our expectations, only an occasional bacterium could be found adhering to the undifferentiated basal cell layer on which the desquamating cells were found. Similarly, *P. aeruginosa* did not adhere to the regenerating mucosa on day 6, in spite of the absence of mature ciliated and serous cells.

(iii) Effect of incubation time on adherence. Adherence of the bacteria to the trachea was increased by prolonged incubation (Fig. 2). Incubation for 15 and 45 min showed similar patterns of adherence. At these times, only about 40% of desquamating cells had bacteria attached. However, after 120 min of incubation about 70% of the desquamating cells had attached bacteria and about 30% had more than 20 bacteria per cell.

(iv) Variability of adherence. One strain of *P. aeruginosa* isolated from the trachea and one from the urinary tract were examined for variability and differences in their adherence patterns (Fig. 3). Considerable variability was observed. The tracheal strain of *P. aeruginosa* adhered to between 38 and 65% of desquamating cells on different days, whereas the urinary strain adhered to 25 to 46% of the desquamating cells. Because of the difficulty in counting the number of bacteria per cell when there were more than 20 per cell, the mean number of bacteria per cell was not calculated.

(v) Strain differences. To look at possible differences between strains of different origins in a more detailed fashion, three strains isolated from the trachea were compared with three strains isolated from the urinary tract. The per-

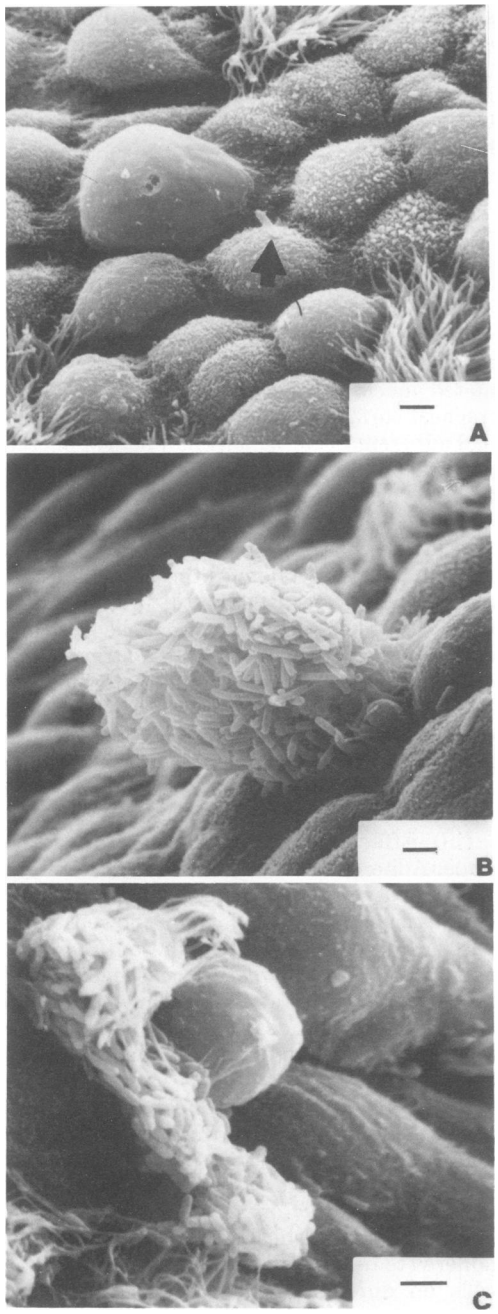

FIG. 1. *Adherence of P. aeruginosa to the mouse trachea. (Each bar represents 2 μm.) (A) Normal mouse trachea showing a single bacterium (arrow) in association with a serous cell. (B) Influenza-infected trachea. Bacteria sometimes adhere to a desquamating cell in masses making visual quantitation difficult. (C) Influenza-infected trachea with large numbers of P. aeruginosa adhering to a desquamating ciliated cell. The adjacent cell does not have adherent bacteria, and the background basal layer has only a few bacteria.*

centage of desquamating cells binding the tracheal strains varied from 35 to 61%, with a mean of 48%, and urinary strains varied from 14 to 36%, with a mean of 22%. These differences were significant ($P < 0.005$, t test). The distribution also appeared different with a greater proportion of tracheal cells having six or more bacteria per desquamating cell (Fig. 4). A mucoid strain of *P. aeruginosa* isolated from a plant was tested, and its adherence was found to be similar to that of the tracheal strains.

Adherence after endotracheal intubation. *P. aeruginosa* colonizes and infects the tracheobronchial tree of patients after endotracheal intubation and of patients with cystic fibrosis. It is not commonly found after influenza virus infections. Therefore adherence studies were done after endotracheal intubation of fer-

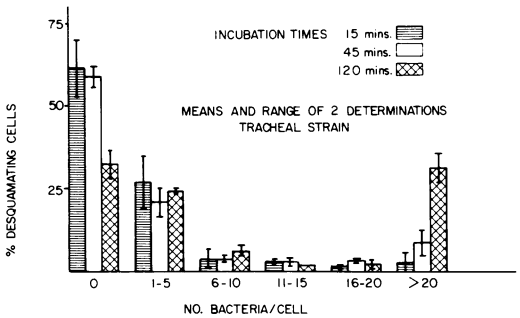

FIG. 2. *Effect of incubation time on adherence. At 120 min, more desquamating cells have adherent bacteria than at 15 or 45 min.*

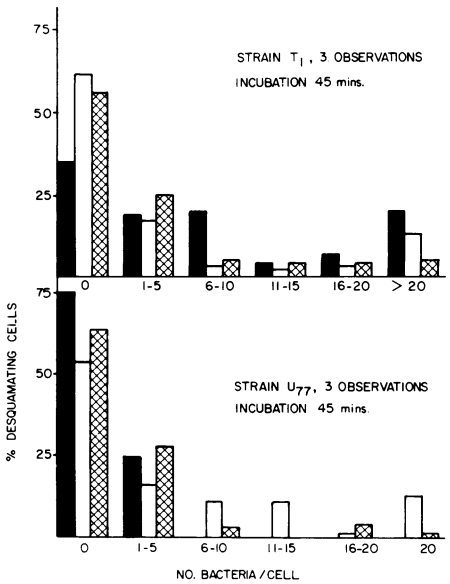

FIG. 3. *Variability of adherence with single tracheal and urinary isolates. Different bars represent experiments done on different days.*

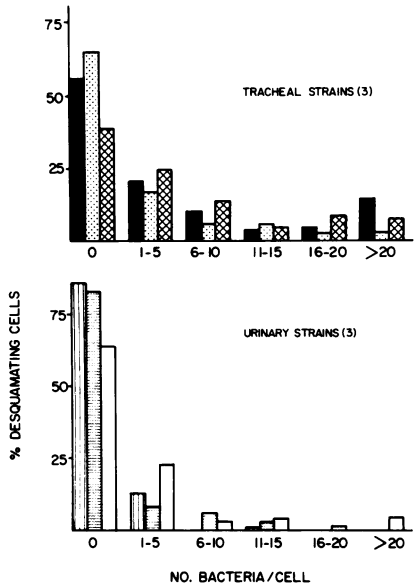

FIG. 4. *Strain differences. Each bar represents a different strain. All experiments were done on the same day. Tracheal strains as a group appear to adhere better than the urinary strains. Incubation time, 45 min.*

rets to see if the same phenomenon occurred; i.e., would the bacteria adhere to injured cells? The ferret tracheal mucosa after intubation is shown in Fig. 5A. There was desquamation, with rounded up tracheal cells as well as erythrocytes on the tracheal surface. The underlying basal layer of cells was visible. After incubation with *Pseudomonas*, bacteria were found adhering only to the loose desquamating cells on the surface (Fig. 5B). Quantitation was not attempted, but three different experiments gave similar findings. Moreover, in all instances, when the basement membrane was exposed, bacteria also adhered to it (not shown). Again, organisms did not adhere to the basal cell layer nor to the intact mature mucosal cells. Three tracheal strains were examined for adherence, and all demonstrated adherence to desquamating cells or basement membrane when it was exposed.

DISCUSSION

These studies show that *P. aeruginosa* adheres to desquamated or desquamating cells of the respiratory tract after influenza virus infection or endotracheal intubation. There was little or no adherence to the normal trachea, the undamaged basal cell layer or to the regenerating layer of cells. After endotracheal intubation, the bacteria also adhered to the basement membrane when this was exposed. The number of desquamating cells with adherent bacteria and the number of bacteria per cell seem to increase with increasing incubation time. All seven strains of *P. aeruginosa* tested showed adherence, but it appeared that strains isolated from the upper respiratory tract adhered better to tracheal cells than did the strains isolated from the urinary tract.

The observation that *P. aeruginosa* did not adhere to the basal and regenerating cells, even though mucociliary activity was absent, suggests that mucinous glycoproteins and ciliary action are not the only mechanisms that prevent the adherence of *P. aeruginosa*. The normal mucosal, basal, and regenerating cells may actually lack binding sites for this organism or, if they have binding sites, these may be hidden by surface components.

Bacterial factors responsible for adherence of this organism are unknown, but there are many

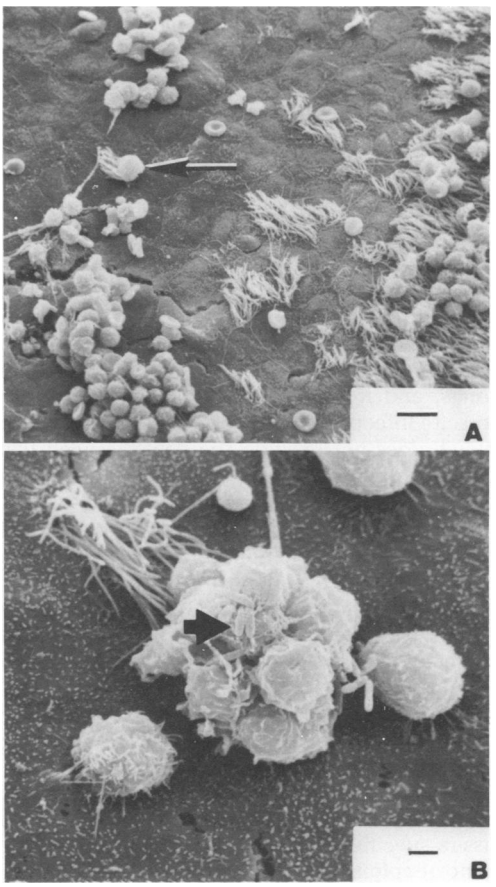

FIG. 5. *Ferret tracheal epithelium. (A) After intubation, showing rounded up desquamating cells (arrow) and erythrocytes on the basal cell layer. Crack in bottom left resulted from specimen preparation. (Bar represents 10 μm.) (B) P. aeruginosa incubated with trachea for 45 min, then washed off. Bacteria adhere only to desquamating cells (arrow). (Bar represents 2 μm.)*

possibilities. Polysaccharides, lipopolysaccharides, glycoproteins, and pili are all present on the surface of this organism. Any of these could mediate adherence. In addition, if host cell damage is important for adherence, then there are potential roles for the exotoxins and proteases produced by the organism in this phenomenon. These extracellular substances may conceivably enhance adherence by their ability to kill cells and modify their surfaces, thus preparing more cells for adherence. However, data on their potential roles in this phenomenon are lacking.

Adherence of bacteria to the respiratory tract may play several roles in disease. Colonization and tracheobronchitis which are seen after endotracheal intubation are probably results of this process. Similarly, surface changes which have been caused by viruses or other bacteria may allow *P. aeruginosa* to colonize the respiratory tracts of cystic fibrosis patients. However, even more important, adherence to desquamating cells could initiate a pneumonia. It is conceivable that bacteria attached to desquamating cells could multiply on these cell surfaces before desquamation is complete. Some of these colonized cells could then be aspirated into the lungs. In this fashion, a greater microbial burden would be presented to the local host defense mechanisms. Desquamating cells may serve as a host-cleansing action to get rid of bacteria in the gastrointestinal and urinary tracts; however, in the case of the respiratory tract, which is a dead end, desquamating cells could go deeper into the lungs, and if they were colonized by bacteria, initiate infection. This is a theoretical possibility which does not exclude the possible need for a concurrent host defense abnormality in the pathogenesis of *Pseudomonas* pneumonia.

Bacterial adherence to host tissues is usually thought to be a specific interaction between the bacterial surface and host cell receptors. This specificity is well demonstrated in the oral cavity, where colonization of the tongue, gums, and teeth by bacteria seem to parallel the adhesive properties of the bacteria for these surfaces (7). Similarly, the adherence of *N. gonorrheae* to primate Fallopian tubes but not to tissues of lower species is thought to be a specific phenomenon which determines the host range of this pathogen (9). Hence, adherence is thought to be tissue specific and to be an important determinant of colonization and pathogenicity. In contrast to specific adherence, our observations suggest that adherence may also be "opportunistic," i.e., it may occur only after tissues are altered in some way. *P. aeruginosa* is not a natural colonizer of animal tissues, nor is it a natural pathogen. It is generally an "opportunistic" organism which causes disease in the compromised or physically manipulated host. Since *P. aeruginosa* is an opportunist, it is not surprising that this organism adheres to host cells only after they have been damaged in some way. Support for our hypothesis has also come indirectly from other work. It has been shown that *P. aeruginosa* adheres better to damaged heart valves than to normal ones (C. H. Ramirez-Ronda, Clin. Res. **26**:404A, 1978), and that there is increased adherence of *P. aeruginosa* to buccal cells after trypsinization of these cells (D. E. Woods, D. C. Straus, W. G. Johanson, Jr., and J. A. Bass, Abstr. Annu. Meet. Am. Soc. Microbiol., 1979, B27, p. 20). In addition, we have recently shown that injuring the cornea facilitates adherence of this organism to the cornea (R. Ramphal et al., Annu. Meet. Acad. Opth., Ocular Micro. Immunol. Group, abstr. no. 3, 1978). Therefore, *P. aeruginosa* does not seem to have the tissue specificity shown by many other pathogens but rather adheres to tissues of different origins after various kinds of injury.

Besides *Pseudomonas* respiratory tract disease, adherence to injured or altered cells may have added clinical significance. Keratitis after corneal injury, burn wound infections, and even colonization of patients receiving cytotoxic chemotherapy may all be consequences of this phenomenon. In summary, we have shown that *P. aeruginosa* adheres to injured tracheal cells and we propose that this opportunistic adherence may be central to the pathogenesis of disease caused by this microorganism.

ACKNOWLEDGMENTS

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